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Human CD43 is a large, abundant and highly charged transmembrane molecule which inhibits intercellular interaction. Normally CD43 expression is restricted to the surface of hematopoietic cells. However, primary breast cancer tumors and their metastases also exhibit CD43 expression. Despite the linkage between breast cancer and abnormal CD43 expression, the molecular mechanisms responsible for this abnormal expression have never been investigated. The goal of this project is to elucidate these mechanisms. In the first year of the project we have shown that the breast cancer cell line MCF-7 exhibits abnormal CD43 expression. In addition, we have demonstrated that *in vitro* the proximal promoter region directs normal expression of the CD43 gene mediated by the transcription factors Pura and hnRNP-K. Our results demonstrate that tissue culture cell lines can be used to test novel strategies for the treatment of breast cancer based on targeting CD43. We have determined that such strategies could now include the specific manipulation of Pura and hnRNP-K expression.

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INTRODUCTION

Human CD43 is a large, abundant and highly charged transmembrane molecule which inhibits intercellular interaction (1 - 3). Normally CD43 expression is restricted to the surface of hematopoietic cells. However, in 1996 it was reported that primary breast cancer tumors and their metastases also exhibit CD43 expression (4).

Expression of CD43 on breast cancer cells which are not hematopoietic in origin is clearly abnormal and suggests a role in cancer development. Indeed, based on the normal function of CD43 on the surface of hematopoietic cells, two such roles can be envisioned. First, by inhibiting cell-cell interaction, CD43 may turn the primary tumor into a loose cellular mass which sheds potentially metastatic neoplastic cells into the circulation. Second, by inhibiting the engagement of cancer cells by immune effector cells such as natural killers and cytotoxic T lymphocytes, CD43 may cause cancer cells to escape immunosurveillance. Such a process of escape is supported by the finding that secretion of CD43 by a human colon adenocarcinoma cell line inhibits natural killer-mediated lysis (5).

Despite the linkage between breast cancer and abnormal CD43 expression, the molecular mechanisms responsible for this abnormal expression have never been investigated. The goal of this project is to elucidate these mechanisms. In this way we will further our understanding of the basic molecular defects which underlie breast cancer and also lay the groundwork for development of alternative treatments based on the specific targeting of these defects.

BODY

It is our hypothesis that abnormal expression of CD43 in breast cancer must be due either to mutation of the gene by which it is encoded and/or alterations in the transcription factors by which this gene is controlled.

Prior to funding by the U.S. Army Medical Research and Materiel Command we isolated the human CD43 gene, determined its structure and demonstrated that its proximal promoter region is responsible for its normal expression in hematopoietic cells (6 - 8). In addition, we demonstrated that the proximal promoter interacts with two unidentified nuclear proteins, MS-2 (9) and MS-A /PyRo1 (8). The goal of the current research project is to extend these preliminary findings to determine the molecular mechanisms responsible for abnormal CD43 gene expression in breast cancer. In order to achieve this goal three tasks are being undertaken. The research accomplishments associated with each of these tasks is described below.

Task 1: Determine the Frequency of Abnormal CD43 Expression in Breast Cancer

Rational: A previous study of nine breast cancer tumors demonstrated that seven exhibited abnormal expression of CD43 (4). While provocative, this finding clearly needed to be extended in order that the true frequency of abnormal CD43 expression in breast cancer can be determined. This we proposed to accomplish by immunocytochemical screening of a broad range of breast tumors and cell lines.

Progress: Using the CD43 monoclonal antibody BS1 (10) we have screened by Western blotting lysates prepared from the breast cancer cell lines Hs578T, MCF-7, SK-BR-3 and T-47D. Also we have screened lysates prepared from the non-tumorogenic breast cell line MTSV1.7 (11). As a positive control for these studies lysates were used from the monocytic cell line U937. As a negative control lysates prepared from the epithelial cell line HeLa were employed. No reactivity with the BS1 antibody was detected for the cell lines MTSV1-7, Hs578T, SK-BR-3 and T-47D. However, reactivity was detected in lysates prepared from MCF-7. The molecular mass of the reactive signal was approximately 40Kd. The molecular mass of the polypeptide backbone of CD43 produced normally in hematopoietic cells is 38.4Kd (6, 12) and, with glycosylation events, CD43 usually exhibits an apparent molecular mass ranging from 115 to 135Kd (13, 14). This large molecular mass we detected in U937 cells. We are currently investigating the nature of the 40Kd protein detected with BS1 in MCF-7 cells. Two outcomes of this investigation are possible. First, MCF-7 may produce a non-glycosylated form of CD43 or, second, MCF-7 may produce a form which is glycosylated but truncated. As well as further analysis of MCF-7 we are screening additional breast cancer cell lines obtained from the American Type Culture Collection (ATCC) for CD43 expression. The results obtained from screening cell lines are being complemented by screening of breast cancer tissue obtained from patients. To date samples have been collected from twenty five such patients. Normal breast tissue from five of these patients has been collected to act as negative controls. Two lymph node tissue samples containing CD43-positive hematopoietic cells have been collected to act as positive controls. We have sectioned the positive and negative control samples and subjected them to immunocytochemical analysis using the BS1 antibody subsequently reacted with FITC labeled anti-mouse immunoglobulin. These analyses have established the conditions were positive signals are obtained in lymph node tissue but no signal is detected in normal breast tissue. We now plan to use these conditions to screen breast cancer tissue samples.

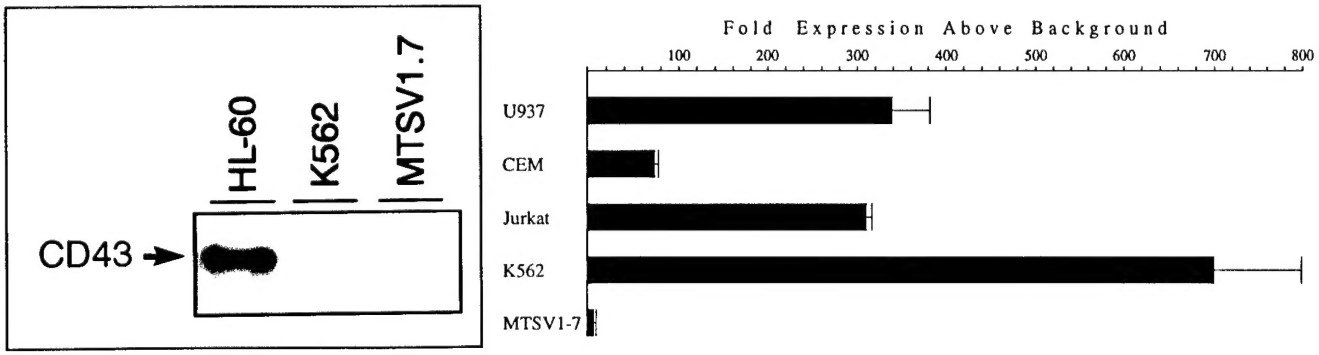
Task 2: Determine CD43 Gene Structure in Breast Cancer

Rational: Abnormal expression of the CD43 gene in breast cancer must be due to its mutation and/or alterations in the transcription factors which regulate its activity. Detection of mutations in the CD43 gene represents Task 2 of the proposed studies.

Progress: Total genomic DNA has been prepared from the breast cancer cell line MCF-7. This line is being analyzed since we have shown it exhibits CD43 expression (see above). We plan to subject the genomic DNA of MCF-7 to Southern blot analysis using a range or restriction endonucleases. This

analysis will establish whether CD43 produced in MCF-7 results from the expression of a CD43 gene which is rearranged. Critical to determining which alterations in CD43 gene structure are of functional significance is a prior determination of which elements within the CD43 gene are responsible for driving its normal pattern of expression. Our preliminary work indicated that the proximal promoter of the CD43 gene extending from nucleotides -2 to +99 drives expression in hematopoietic cells but not in the non-tumorogenic cell line MTSV1-7. This work has been confirmed by repeated experiments using the plasmid p43Wt which contains nucleotides -2 to +99 of the CD43 gene fused to the 5' end of a promoterless luciferase gene. The p43Wt construct was transfected into U937 pro-monocytic cells, Jurkat T-lymphocytes, K562 pre-erythroid/pre-megakaryocytic cells, CEM T lymphocytic cells and the non-tumorogenic breast epithelial cell line MTSV1-7. Measurement of luciferase activity demonstrated that in U937 cells p43Wt directs expression which is 338 fold higher than that directed by the parent plasmid. Such expression in U937 cells compares favorably to the 309 and 698 fold above parental expression levels directed by p43Wt in Jurkat T-lymphocytes and K562 pre-erythroid/pre-megakaryocytic cells, respectively. When p43Wt was transfected into CEM T lymphocytic cells it directed a level of expression only 74 fold above that directed by its parent. This low level of promoter activity compared to other hematopoietic cell types is reflected in the relatively low level of CD43 protein produced by CEM cells (14). CD43 mRNA is undetectable in the epithelial cell line MTSV1-7 (Figure 1). In this cell line p43Wt directs expression only 7 fold above that directed by the parental plasmid. Our findings differ from those of Kudo and Fukuda who reported the proximal CD43 promoter is active when transfected into epithelial cells (15). However, Hela not MTSV1-7 cells were used in these studies. Hela cells are derived from a cervical carcinoma while MTSV1-7 cells are derived from normal breast tissue (11, 16). The development of carcinoma is associated with dramatic changes in DNA methylation (17, 18). Kudo and Fukuda have reported that the tissue-specific expression of the CD43 gene is regulated by DNA methylation (19). Therefore, it is possible that the inappropriate expression of the CD43 promoter when transfected into HeLa rather than MTSV1-7 cells reflects the disruption of the DNA methylation machinery associated with carcinogenesis. Analysis of the methylation state of the CD43 gene in breast cancer will be determined during the course of the present study. In summary, our transfection analysis indicates that the region of the CD43 gene extending from -2 to +99 is sufficient *in vitro* to direct appropriate tissue-specific and cell-specific expression (Figure 1).

Figure 1: Tissue Specific Activity Directed by the Promoter Region of the CD43 Gene. Depicted on the left of the figure is a Northern blot showing the relative levels of expression of CD43 mRNA in the leukocytic cell lines HL-60 and K562 and the breast epithelial cell line MTSV1-7. The construct p43Wt was generated by inserting nucleotides -2 to +99 of the CD43 gene promoter upstream of the promoterless luciferase gene present in the vector pATLuc (20). U937, CEM, Jurkat, K562 and MTSV1-7 cells were transfected with p43Wt in parallel with pATLuc. The right of the figure depicts the level of luciferase activity directed by p43Wt above that directed by pATLuc after correction for transfection efficiency. Each histogram represents the mean ± the standard deviation of three independent experiments.



Task 3: Determine Transcription Factor Structure in Breast Cancer

Rational: Abnormal expression of the CD43 gene in breast cancer must be due to its mutation and/or alterations in the transcription factors which regulate its activity. Detection of alterations in regulatory transcription factors represents Task 3 of the proposed studies.

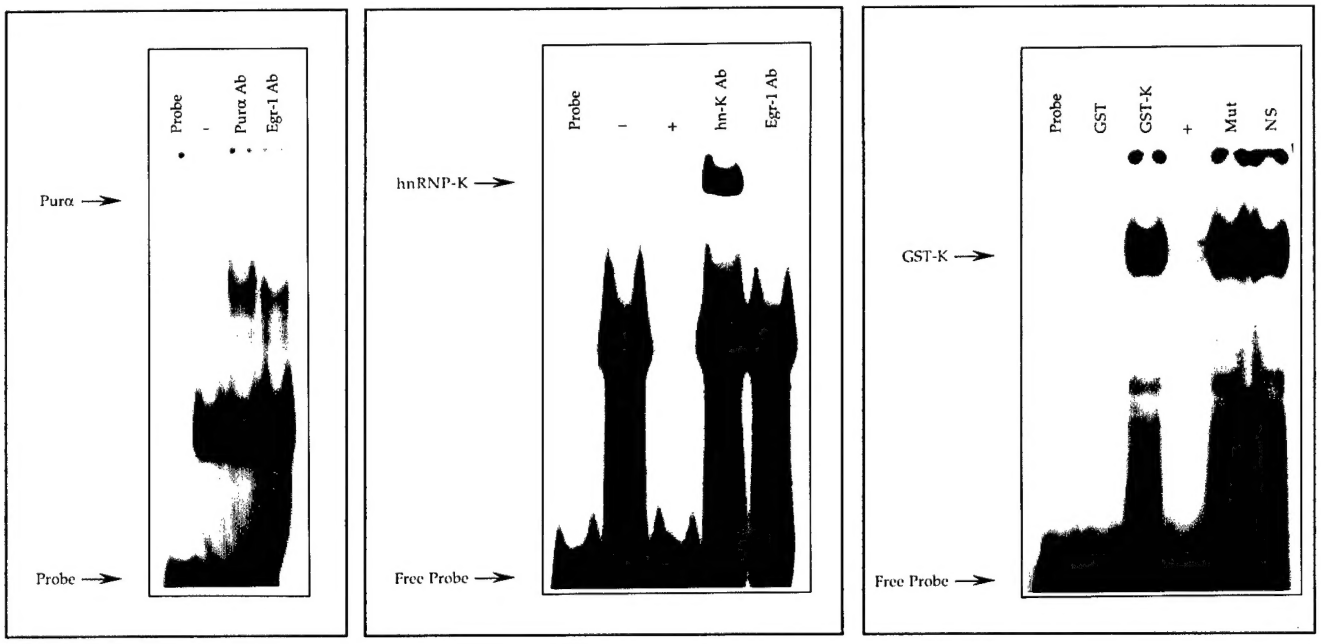
Progress: In order to determine which alterations in transcription factor expression are responsible for abnormal CD43 expression in breast cancer first it must be determined which factors are responsible for driving normal CD43 expression. Studies by other groups have identified the cloned factors Sp1 and MeCP2 as important players in driving appropriate CD43 gene expression (15, 21). Our preliminary data identified the unknown factors MS-2 and MS-A/PyRo1 also as important regulators of normal CD43 expression (8, 9). Since alteration of Sp1, MeCP2, MS-2 and MS-A/PyRo1 expression represents an obvious means of effecting abnormal CD43 expression in breast cancer, it was critical to identify the unknown factors MS-2 and MS-A/PyRo1. This we have achieved.

MS-2 binds the -2/+17 region of the CD43 promoter. Analysis of the nucleotide sequence of the -2/+17 region revealed it contains four copies of the sequence GGN, where N is not G. Repeats of this sequence represent the recognition element of the transcription factor Purα (22, 23). Purα can bind double-stranded DNA but exhibits a ten fold preference for DNA which is single-stranded (23). To determine whether MS-2 was Purα we first attempted to demonstrate whether MS-2 was capable of binding single-stranded DNA. EMSA analysis demonstrated that indeed this was the case (Figure 2, lane marked -). MS-2 binding activity was observed for the sense strand but no factor was found to bind the anti-sense strand (data not shown). Therefore, MS-2 exhibits DNA binding characteristics consistent with those of Purα. Next we determined whether MS-2 interacts with the monoclonal antibody PC12 which specifically recognizes Purα (24). This analysis proved positive demonstrating that MS-2 is immunologically indistinguishable from Purα (Figure 2, lane marked Purα Ab). A control antibody recognizing the transcription factor Egr-1 failed to interact with MS-2 demonstrating the specificity of our immunological detection procedure.

MS-A/PyRo1 interacts with the CD43 promoter in the region spanning nucleotides +18 to +39. We purified MS-A/PyRo1 by affinity chromatography, digested the preparation with trypsin and subjected the resulting peptides to analysis by mass spectrometry. The fragmentation patterns produced from our preparation were then compared to those of known proteins deposited in the Mascot database created by Matrix Science. The most significant match to a known human protein was with Heterogeneous Nuclear Ribonucleoprotein K (hnRNP-K). This factor, like Purα, binds double-stranded DNA but exhibits a distinct preference for binding DNA which is single-stranded (25). We determined that MS-A/PyRo1 binds the single-stranded sense strand of the +18/+39 region of the CD43 gene (Figure 2 , Middle Panel). In addition, we established by electrophoretic mobility supershift analysis using an anti-hnRNP-K antibody that MS-2 is immunologically indistinguishable from hnRNP-K (Figure 2, Middle Panel). The anti-hnRNP-K antibody was obtained from Karol Bomsztyk at the University of Washington (26). A bacterial expression vector capable of producing hnRNP-K linked to glutathione S-transferase (GST) was obtained from David Levens at the National Institutes of Health (25). Recombinant hnRNP-K-GST was produced using this vector and shown to bind a radiolabeled single-stranded oligonucleotide representing the sense strand of the +18/+39 region of the CD43 gene (Figure 2, Right Panel). This binding was effectively competed by an unlabeled excess of the +18/+39 oligonucleotide. However, binding failed to be competed with an identical molar excess of a mutant version of the +18/+39 oligonucleotide. We have shown that this mutant also fails to interact with MS-A/PyRo1 (8).

Figure 2: Demonstration that Purα and hnRNP-K Bind the CD43 Promoter. (Left Panel): A radiolabeled single-stranded oligonucleotide, *43-2/17*, representing nucleotides -2 to +17 of the sense strand of the CD43 gene was incubated with no nuclear extract (Probe) or an extract prepared from the nuclei of U937 cells induced for 24 hours with phorbol ester (PMA). Binding reactions were performed in the absence (-) of a molar excess of competing oligonucleotide, the presence of the monoclonal

antibody 9C12 (Purα Ab) (24) or the polyclonal antibody Egr-1-588 (Egr-1 Ab) (Santa Cruz Biotechnology, Santa Cruz, CA). The complex consisting of probe, Purα and 9C12 is marked with an arrow (Purα) as is the protein-free DNA probe (Probe). (Middle Panel): A radiolabeled single-stranded oligonucleotide, *43-18/39*, representing the sense strand of the +18/+39 region of the CD43 gene was incubated with no nuclear extract (Probe) or a nuclear extract prepared from Jurkat cells. Binding reactions were performed in the absence (-) or presence (+) of a 200 fold molar excess of unlabeled *43-18/39*. Binding reactions were also performed in the presence of the monoclonal antibody 53Ab which specifically interacts with hnRNP-K (hn-K Ab) (26) or the control antibody Egr-1-588 (Egr-1 Ab). Marked with arrows are the free probe (Free Probe) and hnRNP-K bound by probe and 53Ab (hnRNP-K). (Right Panel): Radiolabeled *43-18/39* was incubated with no protein (Probe), 1μg of purified GST (GST) or 1μg of purified GST/hnRNP-K fusion protein (GST-K). Binding reactions containing GST/hnRNP-K were performed in the absence (GST-K) or presence (+) of a 100 fold molar excess of unlabeled *43-18/39*, a mutant version of *43-18/39* which disrupts hnRNP-K binding (Mut) (8) or an unrelated oligonucleotide (NS). The free probe (Free Probe) and probe bound by the GST/hnRNP-K protein (GST-K) are arrowed.



43-2/17: 5′-GGTGGGGTGGGTGGAGCCA-3′ *43-18/39*: 5′-GGGCCCACTTCCTTTCCCCTTG-3′

Mt: 5′-GGGCCCACTTCCTTCATATATG-3′ NS: 5′-GAGTTAGCTCACTCATTAGG-3′

KEY RESEARCH ACCOMPLISHMENTS

1: The breast cancer cell line MCF-7 has been shown to express CD43.

2: The proximal promoter of the CD43 gene has been verified to direct expression in hematopoietic cells but not in the non-tumorogenic breast cell line MTSV1-7.

3: The previously unidentified transcription factor MS-2 which controls expression of the CD43 gene has been identified as Purα.

4: The previously unidentified transcription factor MS-A/PyRo1 which controls expression of the CD43 gene has been identified as hnRNP-K.

REPORTABLE OUTCOMES

Two manuscripts reporting that Purα and hnRNP-K bind and control the expression of the CD43 gene are currently being prepared for submission to peer reviewed journals for publication.

CONCLUSIONS

Our results demonstrate that tissue culture cell lines can mimic the abnormal expression of CD43 detected in human breast cancer tissue. In addition, we have demonstrated that *in vitro* the proximal promoter region directs normal expression of the CD43 gene mediated by the transcription factors Purα and hnRNP-K.

So What: Tissue culture cell lines can be used to test novel strategies for the treatment of breast cancer based on targeting CD43. Such strategies now include the specific alteration of Purα and hnRNP-K expression.

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